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# Molecular cloning of two distinct renin genes from the DBA/2 mouse

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We report the molecular cloning of cDNA copies of DBA/2 mouse submaxillary gland (SMG) renin mRNA, which were used to probe Southern transfers of mouse genomic DNA. The results suggested either that there is a single renin gene containing a large intron in that part of the gene corresponding to the probe, or that there are two distinct renin genes. We have shown that the latter is the case by cloning and isolating two similar but distinct renin genes from DBA/2 mouse DNA. Restriction maps of the regions containing the two renin genes are presented, together with nucleotide sequence data locating a complete exon coding for amino acids 268–315 of mouse SMG renin.

**Key words:** renin/submaxillary gland/mouse/genomic cloning

## Introduction

Renin is an endopeptidase (EC 3.4.99.19) that specifically cleaves angiotensinogen to produce angiotensin I (Cowley *et al.*, 1971). The latter is subsequently cleaved to produce the vasoactive octapeptide angiotensin II, which is involved in the maintenance of normal blood pressure. Although the primary site of renin synthesis is the kidney, renin can also be produced in large amounts by the submaxillary gland (SMG) of the mouse. SMG renin production is under genetic control and is both androgen- and thyroxine-dependent (Wilson *et al.*, 1977, 1981, 1982). Kidney and SMG renins are immunologically and physicochemically similar (Michelakis *et al.*, 1974; Mallin and Poulsen, 1977; Poulsen and Nielsen, 1981), but are secreted under different stimuli. The structure of SMG renin has been determined (Panthier *et al.*, 1982; Misono and Inagami, 1982), and the kidney and SMG renin mRNAs are similar in size and have close sequence homology (Rougeon *et al.*, 1981). Although SMG renin is active in the bloodstream (Bing *et al.*, 1980), the release of large quantities from the gland leads to no significant increase in blood pressure (Bing and Poulsen, 1979). The relationship between the kidney and SMG renins and the physiological function of the submaxillary enzyme are unknown.

Inbred mouse strains can be divided into two groups having significantly different levels of SMG renin. This genetic variation is due to a single gene, *Rnr*, located near the *Pep-3* locus on chromosome 1 (Wilson *et al.*, 1978). The SMG renins of high and low producers differ in their thermostability and immunological properties, suggesting that two distinct proteins are involved and that the *Rnr* locus is either identical with or closely linked to a gene that influences the

structure of the renin molecule (Wilson and Taylor, 1982). Recently, Piccini *et al.* (1982) reported that restriction endonuclease digests of the DNA of high and low renin strains are characteristically distinct and suggestive of a *Rnr* gene duplication in high renin strains.

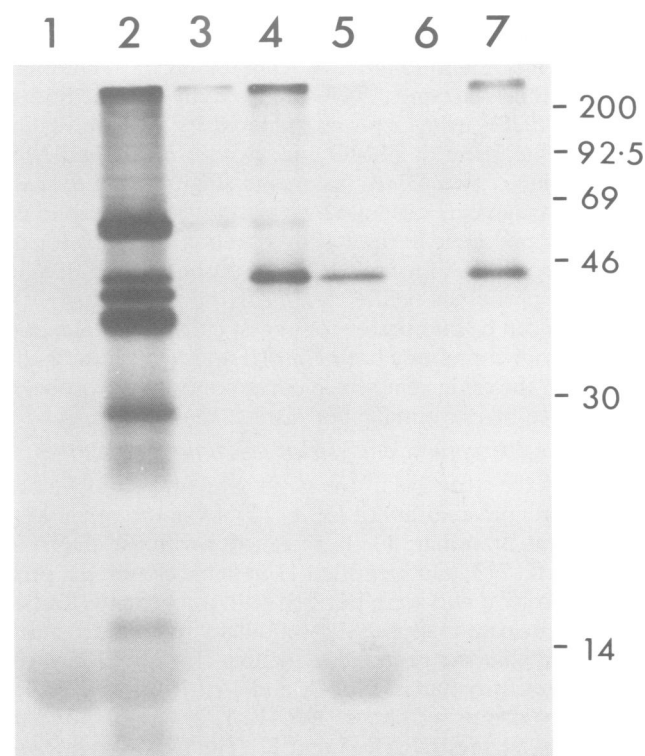
We report the presence in the DBA/2 mouse, a high renin-producer, of two renin genes that show substantial homology in both coding and non-coding regions.

## Results

### Isolation and identification of renin cDNA clones

The cell-free translation product of SMG renin mRNA was identified by immunoprecipitation with mouse SMG renin antiserum (kindly supplied by K. Poulsen). Figure 1 shows the precipitation of the renin precursor, a 44-K polypeptide (Panthier *et al.*, 1982).

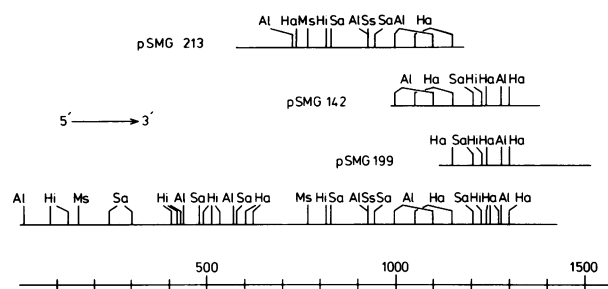
An SMG cDNA library was constructed in the vector pAT153 as reported (Skup *et al.*, 1982). Recombinant plasmids were screened for their ability to hybridise to renin



**Fig. 1.** Immunoprecipitation of the renin precursor from the translation products of total SMG mRNA and mRNA selected by hybridization to a renin cDNA clone. **Lane 1**, control translation with no added mRNA; **lane 2**, translation products of total male DBA/2 SMG mRNA; **lanes 3** and **4**, immunoprecipitation of the translation products of total mRNA (**lane 2**) with normal rabbit serum and renin antiserum, respectively. **Lane 5**, translation products of mRNA selected by hybridization to pSMG 142; **lanes 6** and **7**, immunoprecipitation of the translation products of pSMG 142 selected mRNA (**lane 5**), with normal rabbit serum and renin antiserum respectively. **Lanes 1, 2, and 5** show 3  $\mu$ l of sample. **Lanes 3, 4, 6, and 7** show the immunoprecipitate from 10  $\mu$ l of translation mix. The sizes of mol. wt. markers are given in kilodaltons.

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**Fig. 2.** Restriction maps of renin cDNA clones pSMG 213, pSMG 142, and pSMG 199. The maps are drawn relative to the restriction map of a full length cDNA, which was derived from a computer search of the DNA sequence determined by Panther *et al.* (1982). The scale is drawn in base pairs from the 5' end of the sequence mentioned above. Symbols for restriction enzymes are as follows: Al = *AluI*; Hi = *HinfI*; Ms = *MspI*; Sa = *Sau3A*; Ha = *HaeIII*; Ss = *SstI*.

mRNA, assayed by *in vitro* translation of the selected mRNAs and immunoprecipitation of their products (Figure 1). Clone pSMG 142, identified as a renin clone, was then used to re-screen the complete cDNA library. Figure 2 shows the restriction maps of three renin cDNA clones, pSMG 199, pSMG 142, and pSMG 213, indicating their position with respect to the renin cDNA sequence of Panther *et al.* (1982).

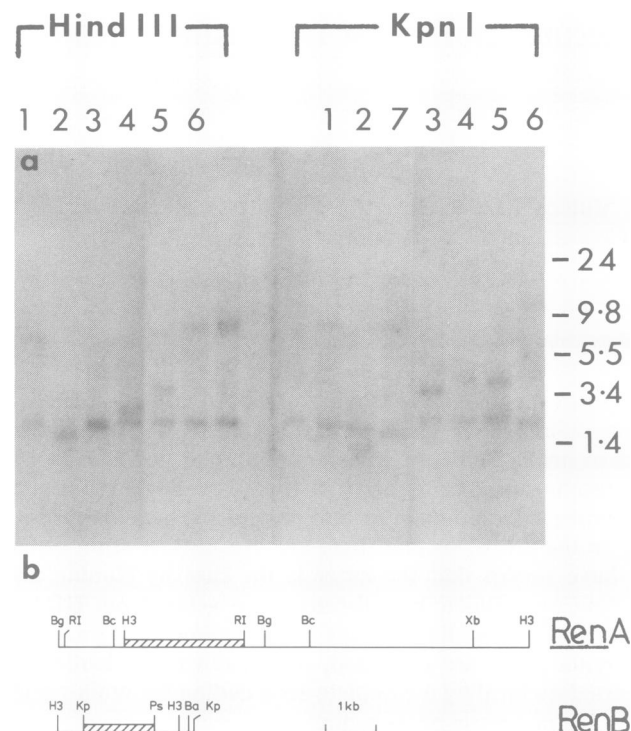
#### Southern analysis of genomic DNA

The structure of the renin gene(s) was analysed by digesting DBA/2 mouse DNA with restriction endonucleases. Digests of DBA/2 DNA with *HindIII* or *KpnI*, singly or in combination with other enzymes, were analysed by the method of Southern (1975), using a probe prepared by nick-translation of the cDNA insert of pSMG 142. Both *HindIII* and *KpnI* alone produced two DNA fragments (Figure 3). From the pattern of fragments generated when cut with the second enzyme, it was possible to draw simple restriction maps for the two regions of DNA recognised by the probe (Figure 3). Since the cDNA probe has neither a *HindIII* nor a *KpnI* site, the above data can be interpreted to suggest either the presence of a large intron, containing both *HindIII* and *KpnI* sites, within that part of the renin gene which corresponds to the probe, or the existence of two similar but distinct renin genes.

#### Isolation and structural analysis of genomic renin clones

To study the structure of the renin gene (or genes) we constructed a genomic library of DBA/2 DNA in the vector  $\lambda$ L47 (Loenen and Brammar, 1980), screened it with the cDNA insert of pSMG 213, and identified 11 possible clones. Six proved to be positive and were purified to homogeneity. Restriction endonuclease mapping showed all six to be independent isolates and that the genomic sequences cloned fell into two classes, arbitrarily named *Ren-A* and *Ren-B* (Figure 4). The *Ren-A* class comprises phages *DBARn1*, 2, 6, and 7 and the *Ren-B* class, phages *DBARn5* and 11. The *Ren-A* and *Ren-B* regions in these isolates cover 21 kb and 14 kb, respectively. The *Ren-A* phage DNAs overlap in a region that corresponds to the promoter-distal section of the *Ren-A* gene, since it hybridizes to the pSMG 213 cDNA clone derived from the 3' end of the SMG renin mRNA. Comparison of the restriction maps derived for the two genomic regions, *Ren-A* and *Ren-B*, indicates their substantial homology and the position of an insertion or deletion event.

This conclusion was confirmed by the analysis of heteroduplexes formed between the DNAs of  $\lambda$ *Ren-A* and  $\lambda$ *Ren-B* phages (Figure 5). Heteroduplexes of  $\lambda$ *DBARn1*/ $\lambda$ *DBARn11*,

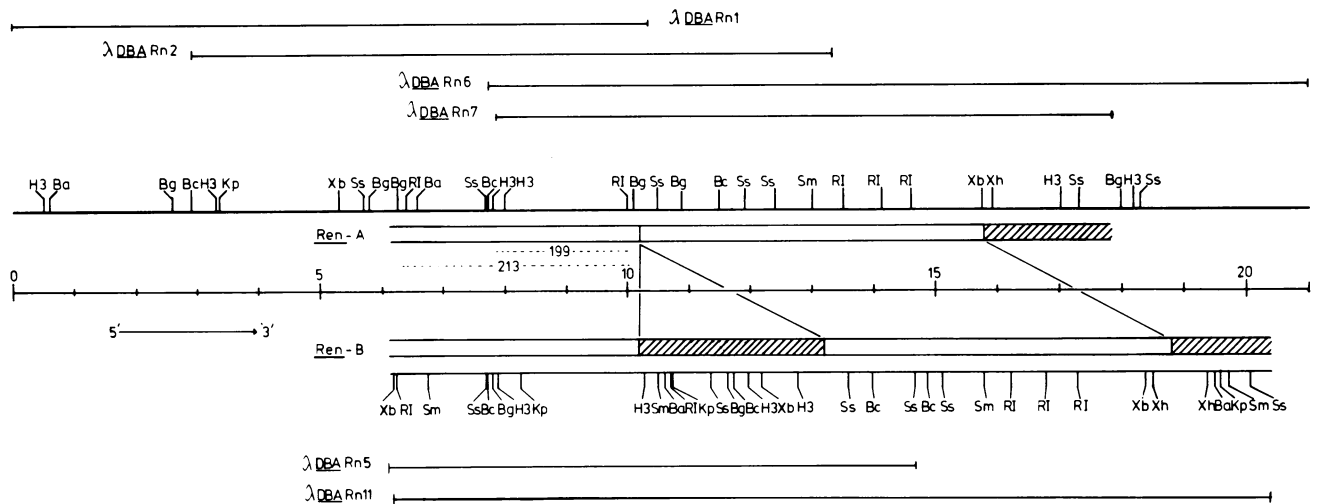


**Fig. 3.** Analysis of DBA/2 mouse DNA by Southern transfer and hybridization to the renin cDNA clone pSMG 142. (a) Restriction digests of liver DNA were run on an 0.8% agarose gel, transferred as described in Materials and methods, and probed with  $^{32}$ P-labelled pSMG 142. DNA was cleaved with either *HindIII* or *KpnI* and then, where indicated, recut with a second enzyme. Restriction enzymes are numbered as follows: 1 = *XbaI*; 2 = *PstI*; 3 = *EcoRI*; 4 = *BglII*; 5 = *BclI*; 6 = *BamHI*; 7 = *HindIII*. (b) Restriction maps of the two regions of DNA hybridizing to the probe pSMG 142 as determined from the Southern transfer data above (a). The regions of genome homologous to the probe must be within the hatched areas. The sizes of mol. wt. markers are given in kilobase pairs. The two homologous regions are designated *Ren-A* and *Ren-B* to show their correspondence with the maps derived from renin genomic clones (see Figure 4).

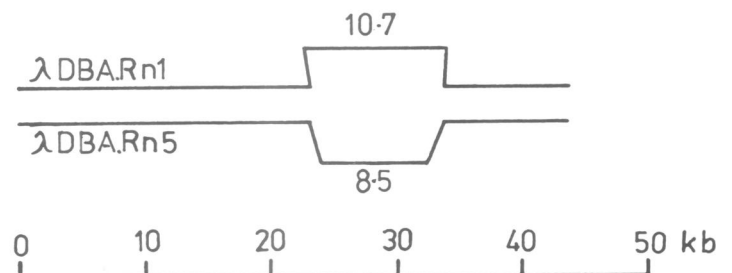
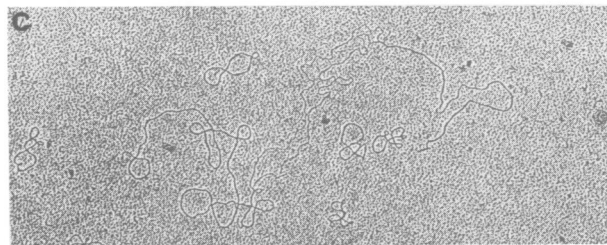
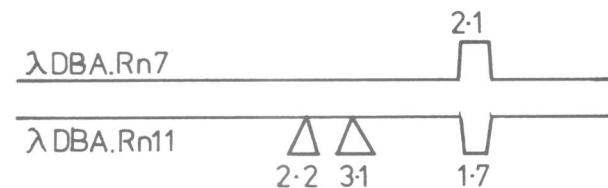
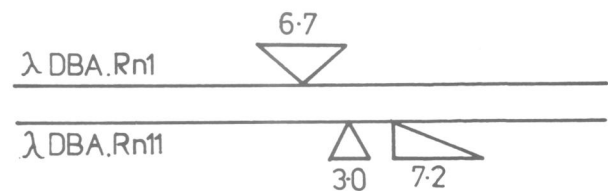
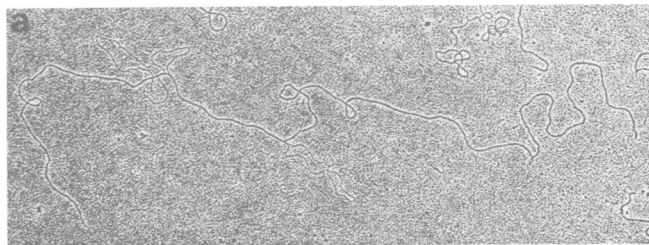
$\lambda$ *DBARn2*/ $\lambda$ *DBARn11*, and  $\lambda$ *DBARn6*/ $\lambda$ *DBARn5* all showed extensive duplex formation and three deletion loops (Figure 5). The central loop contained ~3 kb of DNA in each case and the two terminal loops of varying size represent the non-overlapping regions at the opposite ends of the cloned DNA. Heteroduplexes between  $\lambda$ *DBARn7* and  $\lambda$ *DBARn11* also showed a central deletion loop of ~3 kb of DNA, but one of the terminal loops was of the substitution type, showing that the DNA sequence at one end of the cloned DNA in these two recombinants was non-homologous. The third type of heteroduplex molecule was seen when DNA of  $\lambda$ *DBARn1* was paired with that of  $\lambda$ *DBARn5*; the entire central region appeared as a substitution loop. This is the behaviour expected if the cloned sequences were in opposing orientations relative to the flanking regions of the phage genome. The structures of the *Ren-A* and *Ren-B* regions of the DBA/2 mouse genome, as deduced from restriction mapping and heteroduplex analysis are summarised in Figure 5.

#### Orientation of the renin genes

The appropriate positions of the renin-coding sequences and their orientations with respect to the restriction maps were determined by investigating the homology of different restriction fragments with cDNA probes made from pSMG 199 and pSMG 213. The former recombinant contains se-



**Fig. 4.** Physical maps of the regions containing the renin genes. Restriction maps were derived from digests of six recombinant phage DNAs. The extents of the regions cloned in each phage are shown at the top and bottom of the figure. The double lines between the restriction maps summarise the data from heteroduplex mapping, showing homologous (—) and non-homologous (▨) regions. The accuracy in the placement of the ends of the homologous regions by heteroduplex mapping is  $\pm 200$  bp, so that it is likely that the *Xba*I and *Xho*I targets at co-ordinate 16 kb in the *Ren-A* map and 18.5 kb in *Ren-B* are within the homologous sequences. The dotted lines show the DNA fragments that hybridize to the pSMG 199 and pSMG 213 cDNA probes and allow the orientation of the coding sequences to be deduced. The central scale is graduated in kilobase pairs. Symbols for restriction enzymes are as follows: Ba = *Bam*HI, Bc = *Bcl*I, Bg = *Bgl*II, H3 = *Hind*III, Kp = *Kpn*I, RI = *Eco*RI, Ss = *Sst*I, Xb = *Xba*I, Xh = *Xho*I.



**Fig. 5.** Heteroduplex mapping of the genomes of recombinant phages carrying mouse renin genes. Heteroduplex DNA molecules formed between DNAs from (a)  $\lambda$ DBA.Rn1 and  $\lambda$ DBA.Rn11, (b)  $\lambda$ DBA.Rn7 and  $\lambda$ DBA.Rn11, and (c)  $\lambda$ DBA.Rn1 and  $\lambda$ DBA.Rn5 are shown. The line drawings give the sizes and positions of the deletion and substitution loops derived from measurements of  $\sim 20$  molecules.

quences corresponding to  $\sim 375$  nucleotides at the 3' end of the SMG renin mRNA, while pSMG 213 extends another 450 nucleotides towards the 5' end. The pSMG 199 probe hybrid-

ised with the 2.2-kb *Hind*III/*Eco*RI fragment from coordinates 8–10 kb of the *Ren-A* series of phages, but showed no detectable homology with flanking fragments. It also

hybridised to the 2.6-kb *Hind*III fragment from the corresponding region of the *Ren-B* phages. The larger probe prepared from pSMG 213 hybridised to these same fragments, but also picked out the 1.45-kb *Eco*RI/*Hind*III fragment from co-ordinates 6.4–7.9 of the *Ren-A* phages 1 and 2, as well as the corresponding fragment from the *Ren-B* phage 11. These data show that both the *Ren-A* and *Ren-B* renin genes are oriented so as to be read from left to right on the maps in Figure 4. The promoter-distal ends of the renin genes must be located within the 8–10 kb regions of both maps, but we are unable to locate the promoter-proximal ends of the genes from these data. The cDNA inserts in plasmids pSMG 199 and 213 do not contain *Hind*III targets and neither probe hybridised to the small *Hind*III fragment at co-ordinate 8 of the *Ren-A* region, suggesting that this region of the genomic map must constitute an intron.

The presence of neighbouring *Sst*I and *Bcl*I sites at coordinate 7.8 kb from the left end of the *Ren-A* genomic map was consistent with this region's correspondence to the 3' end of the renin mRNA sequence, as these restriction sites are

The sequence (Figure 6) confirmed the orientation and position of the *Ren-A* gene and revealed an exon closely corresponding to the published sequence of the SMG renin cDNA, between nucleotides 841 and 985 (Panthier *et al.*, 1982). This exon is flanked by two sequences not found in the cDNA and is bounded by consensus intron/exon junctions (Lewin, 1980). Within the region of homology to the cDNA clone of Panthier *et al.* (1982) the sequence shows 96% identity. Five bases differ, at the positions indicated in Figure 6, though two of these alterations lead to no change of amino acid in the protein coded.

Molecular cloning of DNA copies of the mouse mRNA for SMG renin has provided a probe for the analysis of renin genes and for the isolation of genomic clones. Restriction en-

zyme mapping of the cloned genomic DNA correlates well with the Southern mapping data and establishes the absence of detectable sequence rearrangement during the cloning procedures. The mapping data prove the existence in the DBA/2 mouse genome of two regions having extensive homology with the renin-coding sequence. We cannot be certain that these two regions both include intact renin genes, since without complete nucleotide sequences the possibility remains that one sequence could be incomplete or represent a pseudogene. For ease of reference and to avoid confusion with existing genetic nomenclature for mouse renin genes we have called the two renin-related sequences *Ren-A* and *Ren-B*.

The homology between the *Ren-A* and *Ren-B* regions of the genome extends over at least 10 kb of DNA and stretches well beyond the renin genes. Within the large duplicated region there has been a deletion or insertion of 3 kb of DNA, downstream of the renin-coding region. The region of homology could be >10 kb, since the six recombinant phages studied have not defined the left end of the homologous region.

Although the extended homology between *Ren-A* and *Ren-B* would allow the inclusion of multiple copies of the renin gene in each chromosomal region, the Southern mapping of the DBA/2 chromosomal DNA makes this possibility very unlikely. A double digest of chromosomal DNA with *HindIII* and *PstI* yields only 1.9-kb fragments hybridising with the cDNA probe. After digestion with *KpnI* plus *PstI*, the only fragment from the *Ren-B* region that is revealed by the probe is 1.4 kb long. Since the renin mRNA sequence is ~1.5 kb long, and the cDNA insert in the pSMG 142 probe is ~400 bp, it is very unlikely that the 1.4-kb fragment contains two copies of the region homologous to the probe. The observations are strongly suggestive of there being only one renin gene in each of the *Ren-A* and *Ren-B* regions.

The renin level in the SMG of the mouse is governed by a regulatory locus, *Rnr*, mapped to chromosome 1 (Wilson *et al.*, 1978). Piccini *et al.* (1982) have recently shown, by Southern transfer analysis with a renin cDNA probe, that a specific DNA polymorphism is intimately associated with the allele at the *Rnr* locus and have suggested that high-producer strains carry a duplication of the renin gene. Our data show that one high renin-producing strain, DBA/2, does have two renin genes, though it remains to be established that they are both active. The genomic mapping of Piccini *et al.* (1982) shows that *KpnI* generates fragments of 20 kb and 2 kb from DBA/2 DNA, but produces only the larger fragment from the DNA of C57BL/6J, a low renin strain. Our data show that the 2-kb *KpnI* fragment derives from the *Ren-B* region and that the *Ren-A* renin gene is contained within a *KpnI* fragment of >18 kb. Taken together, these findings suggest that the low renin strain C57BL/6J contains the *Ren-A* renin gene but does not carry the *Ren-B* copy.

Renin mRNA can be detected in both the SMG and the kidney of low producer strains, including C57BL/6J (Rougeon *et al.*, 1981; J.J. Mullins, unpublished data). Since such strains only appear to contain the *Ren-A* copy of the renin gene, it is evident that this gene must be expressed in both tissues.

As discussed by Piccini *et al.* (1982), the presence of an extra copy of the renin gene in high producers cannot account for the 100-fold elevation in SMG renin levels by mere gene dosage. In order to understand the molecular basis for the variation in renin levels it will be necessary to correlate the ex-

pression with the structures and sequences of the renin loci from high and low renin-producing strains of mice.

## Materials and methods

### Animals

Mice (DBA/2) were supplied by Bantam and Kingman Ltd., Hull, UK.

### Isolation and translation of mRNA

10 week-old male DBA/2 mice were killed by cervical dislocation. SMGs were removed and placed immediately into liquid nitrogen. RNA was prepared essentially by the method of Noyes *et al.* (1979) and poly(A)<sup>+</sup> RNA was purified by oligo(dT)-cellulose chromatography. mRNA was translated in a nuclease-treated reticulocyte lysate prepared according to Pelham and Jackson (1976), containing the following: 20  $\mu$ M each amino acid (minus methionine), 1 mM ATP, 0.2 mM GTP, 80 mM KCl, 2 mM magnesium acetate, 10 mM Tris-HCl pH 7.6, 2 mM glucose, 7 mg/ml creatine phosphate, 50  $\mu$ g/ml calf liver tRNA, 1 mCi/ml [<sup>35</sup>S]methionine (specific activity 1160 Ci/mmol, Amersham International), and 50  $\mu$ g/ml creatine phosphokinase. Immunoprecipitation was carried out by a modification of the method of Dobberstein *et al.* (1979). Translation products and immunoprecipitates were analysed by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) and visualised by fluorography.

### Construction of recombinant plasmids

Recombinant plasmids containing cDNA copies of SMG poly(A)<sup>+</sup> RNAs were made as described by Skup *et al.* (1982). Colony hybridization was performed according to Young *et al.* (1977) and plasmid DNAs were prepared by equilibrium centrifugation in the presence of ethidium bromide (Windass and Brammar, 1979).

### Positive hybridization-translation assays

Positive hybridization was performed by the method of A. Jeffreys (unpublished data) and the selected RNAs assayed by translation and immunoprecipitation of the products (see above).

### Preparation and Southern blotting of mouse DNA

DNA was isolated, transferred, and probed as described by Jeffreys *et al.* (1980), the final wash being carried out in 0.5 x SSC. Nick-translation of DNA was performed by the method of Jeffreys *et al.* (1980).

### Isolation of renin genomic clones

A DBA/2 mouse genomic library was constructed by cloning mouse liver DNA which had been partially digested with *Sau3A*, into the high capacity lambda vector  $\lambda$ L47 (Loenen and Brammar, 1980). Partial *Sau3A* digests of genomic DNA were fractionated on a 10–40% sucrose gradient and fractions in the size range 10–18 kb were pooled. Left and right arms of  $\lambda$ L47 DNA were prepared from a *BamHI/SalI* double-digest and fractionated on a 10–40% sucrose gradient. Fractions containing the 24 and 10 kb left and right arms were pooled.  $\lambda$ L47 arms were annealed and ligated to genomic DNA and the ligation mixture was packaged *in vitro* into phage particles as described by Jeffreys *et al.* (1981). The yield of recombinant phages was  $2 \times 10^6$  p.f.u./ $\mu$ g of genomic DNA, with an average input size of ~10 kb.

Approximately  $8 \times 10^5$  phage were screened by the method of Benton and Davis (1977) using the cDNA probe, pSMG 213. Initial screening was carried out on the host DB102 (*metB*, *supE*, *supF*, *hdsR*, *trpR*, *tonA*) while subsequent purification and propagation was done with a *recBC* host, ED8910 (*supE*, *supF*, *hdsR*, *recB21*, *recC22*).

### General techniques

The methods for manipulation of phages, preparation, restriction, and ligation of DNAs and gel electrophoresis of DNA fragments are described in Burt and Brammar (1982).

### Southern blotting of genomic clones

Gels were cut to size and prepared for transfer according to Wahl *et al.* (1979) and then blotted by the method of Southern (1975). Hybridization was carried out under the conditions described by Jeffreys *et al.* (1980), using  $7 \times 10^6$  c.p.m./ml of nick-translated probe DNA. The final wash was with  $1 \times$  SSC at 65°C.

### DNA sequencing

Fragments used for sequencing were derived from fragments of the genomic clones, subcloned into pAT 153; nucleotide sequencing was performed according to the procedure of Maxam and Gilbert (1980).

### Electron microscopy

DNA heteroduplex molecules were prepared by the methods described by Davis *et al.* (1971), using DNAs at ~15  $\mu$ g/ml in 0.1 M Tris, 0.01 M EDTA pH 8.5, and 50% formamide. DNAs were denatured for 1 min at 70°C and

renatured for 5 h at 37°C. The hyperphase contained 40% formamide and the hypophase 10% formamide, 0.01 M Tris, 10  $\mu$ M EDTA pH 8.5. The grids were Pt/Pd-shadowed and visualized in a Jeol 100 cx electron microscope at an accelerating voltage of 60 kv and a magnification of 8500 x. Measurements were taken from prints using a Kontron digitizing tablet and computer.

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